An Investigation of the Larval Development of *Dirofilaria immitis* (Leidy) in Fleas

By

EDMUND BRUNO STUEBEN

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
August, 1953

ACKNOWLEDGEMENTS

It is hardly adequate to merely acknowledge the Chairman of my Supervisory Committee, Dr. Lewis Berner, but rather I wish to express my deep gratitude and appreciation for his guidance and aid. His sincere efforts have been a constant source of encouragement during the three years of my investigation.

My appreciation is also extended to Dr. C. F. Byers for fulfilling the role of Chairman during Dr. Berner's entomological survey trips to Africa upon the request of the British Government.

I acknowledge special indebtedness to: Dr. W. V. King, former chief, Mr. W. C. McDuffie, present chief, and Mr. P. N. Mason of the United States Department of Agriculture, Bureau of Entomology and Plant Quarantine Research Laboratory at Orlando, Florida, for supplying colonies of fleas and showing me rearing techniques;

Dr. E. Voss, Dr. L. E. Fox, Mr. B. C. Barnes, and Mr. F. R. Thompson of the Department of Pharmacognosy and Pharmacology of the University of Florida for help in surgical techniques and use of their facilities;

The many friends, acquaintances, School of Pharmacy, and the Gainesville Humane Society for allowing me to collect fleas and blood samples from dogs in their care and to perform autopsies on dogs destroyed because of extensive injury or disease;

The College of Arts and Sciences of the University of Florida

for a Graduate Fellowship for the period of June through August 1953;

To my wife, Gisela, for her assistance, diligence with this manuscript, and above all--her patience.

TABLE OF CONTENTS

TABLE OF CONTENTS--Continued

LIST OF TABLES

LIST OF PLATES AND FIGURES

LIST OF PLATES AND FIGURES--Continued

INTRODUCTION

General Distribution of Heartworms in Dogs

The adult filariae of Dirofilaria immitis Leidy (1856) seem to have been observed for the first time during the 17th century by Panthot (1679). La Peyronnie (1778) recorded similar observations a century later. These findings were confirmed at the start of the 19th century by Peysson (1806), Zeviani (1809) and Bobe-Moreau (1813) and soon thereafter the worm was recorded in Europe as far north as Denmark and far south as Italy. Leidy (1856) and Schuppert (1858) noted its presence in the United States. Somerville (1875) reported that an exceptionally high incidence of infection of dogs occurred in Japan and China, while Silva Aranjo (1878) added Brazil to the growing list of countries in which the worm occurs. All of these studies were based on autopsy findings and show that D. immitis infections are apparently world-wide.

Canine infections with the heartworm, D. immitis, are causing increasing concern to dog owners throughout the Atlantic Coast states and Gulf states. Because of the studies of Blackberg and Ashman (1930) and Hinman and Baker (1936), D. immitis is known to be much more prevalent in the South than was commonly suspected. These authors reported that almost 50 percent of the adult dogs from New Orleans

are infected with heartworm.

Review of Theory of Mosquito Transmission

In most instances, the transmission of the worm by any particular vector is correlated with the ability of the prelarval microfilaria* to develop to the infective stage. The theory of mosquito transmission of canine filariasis has been considered so firmly established since Grassi (1890) observed filariae larvae in Anopheles maculipennis, which he then determined to be developmental forms of D. immitis, that searches for other arthropod vectors has been largely neglected. Laboratory experiments have demonstrated that twenty-six species of mosquitoes have been shown to be susceptible to infections with D. immitis. These are as follows:

Species	Place of Investigation	Authority
Aedes aegypti	(United States)	(Hu 1931)
A. canadensis	(United States)	(Hu 1931)
A. caspius	(Italy)	(Grassi & Noe 1900)
A. cinerius	(United States)	(Yen 1938)
A. excrucians	(United States)	(Phillips 1939)
A. infirmatus	(United States)	(Summers 1943)
A. koreicus	(China)	(Feng 1930)
A. sollicitans	(United States)	(Summers 1943)
A. stimulans	(United States)	(Yen 1938)
A. taeniorhynchus	(United States)	(Hu 1931)
A. triseratus	(United States)	(Phillips 1939)
A. vexans	(Italy)	(Grassi & Noe 1900)

*The term prelarva refers to that stage in the life history of the worm prior to its first molt.

Species	Place of Investigation	Authority
Anopheles algeriensis	(North Africa)	(Grassi & Noe 1900)
A. bifuracatus	(Italy)	(Grassi & Noe 1900)
A. crucians	(United States)	(Summers 1943)
A. hyrcanus	(Italy)	(Grassi & Noe 1900)
A. hyrcanus var. sinensis	(China)	(Feng 1930)
A. maculipennis	(Italy)	(Grassi & Noe 1900)
A. punctipennis	(United States)	(Hu 1931)
A. quadrimaculatus	(United States)	(Phillips 1939)
A. superpictus	(Italy)	(Grassi 1900)
Culex fatigans	(Philippine Islands)	(Del Rosario 1936)
C. pipiens	(Italy)	(Grassi & Noe 1900)
C. skusii*	(Australia)	(Bancroft 1901)
C. tarsalis	(United States)	(Yen 1938)
C. territans	(United States)	(Hu 1931)

The species of mosquitoes chiefly responsible for transmission of this disease in nature are not known.

Hinman (1935) has demonstrated that Aedes aegypti, previously thought to be a suitable vector of the worm (Hu, 1931), is in reality not a compatible host. Additional work has shown that those species of mosquitoes susceptible to the developmental stages of the heartworm larvae have a high mortality rate and cannot serve as a suitable vector. It, therefore, became apparent that some other arthropod must be incriminated as a prime transmitter.

With the realization that almost all work on D. immitis dealt with the parasite in the definitive host, it became apparent that the effects of the worm on the intermediate host had been relatively neglected. Earlier work had established the fact that an insect vector

---

*This species may have been misidentified and may actually be C. fatigans.

was required for the intermediate stages of the worm and mosquitoes had been incriminated and fleas suspected. It appeared, therefore, that an investigation of the flea as an intermediate host, with particular emphasis of the effects of the parasite on the insect, was needed to help complete the knowledge of this important parasite. With these requirements in mind, the present investigation was undertaken.

THE PROBLEM

Primary Objectives

The primary objectives of this investigation were to determine:

(1) The incidence of infection of D. immitis larvae in fleas.

(2) The metamorphosis of D. immitis larvae in fleas.

(3) The effect of D. immitis larvae on the mortality rate of fleas.

(4) The effect of D. immitis larvae on the fertility of fleas.

(5) The behavior of D. immitis larvae in fleas.

Secondary Objectives

The secondary objectives were to establish:

(1) The incidence of infection of the definitive host in North Central Florida.

(2) Is any particular breed of dog more apt to have the disease than another?

The secondary objectives were a natural outgrowth of the primary ones. During my investigation of the literature early in the study, I encountered conflicting statements with regard to the incidence of infections in long-haired as opposed to short-haired

dogs; I, therefore, became interested in determining whether or not there was a real correlation. Hall, Price and Wright (1934) had claimed that short-haired dogs are more susceptible to heartworms than long-haired dogs, probably because they have less protection from mosquito bites. Other workers such as Augustine (1936) and Hinman and Baker (1936) found the highest incidence of infection among long-haired dogs. Brown (1939), having discovered slightly heavier infections in long-haired dogs, suggested that fleas and lice infested dogs more successfully when the hair was long and they should naturally be suspected as being intermediate hosts of D. immitis. A brief examination by this author of a small sample of Ctenocephalides felis showed that perhaps his supposition was correct as he found prelarvae, which he did not attempt to identify, in several of them.

The study began to enlarge as the correlations between the primary and secondary objectives interdigiated until it became rather difficult to separate one set from the other. I have attempted, however, in the following discussion to make a clear distinction between the two sets.

TECHNIQUES

Histological Methods

I resorted to serial sectioning of fleas so that I could accurately plot the migration and location of both the prelarvae and developing larvae of D. immitis in the infected flea.

I soon found that when I used a standard fixative such as Bouin's followed by routine dehydration and paraffin embedding that the resultant serial sections were not satisfactory. The stained serial sections of the fleas revealed that the small traces of picric acid that had not washed out interfered with the staining. The exoskeleton in almost all cases had not been sufficiently softened for sectioning and consequently fragmented during this procedure. The shattering of the exoskeleton distorted and ripped many of the internal structures to the extent that identification of these became a problem in itself.

Several of the procedures outlined in the literature were tried; however, my results were never more than fair. Perhaps Gray (1952, p. 14) gave the best summary when he said:

> No really satisfactory method for softening chitinous materials has yet been discovered.

> Insects and other chitinous forms are fixed in the fluid of Carnoy and Lebrun and are transferred without washing to Jurray's mixture where they remain from 12 to 24 hours. Then this mixture is washed out in chloroform and the objects are embedded in paraffin.

Slifer (1933) was very successful in sectioning ticks using the Carnoy and Lebrun fluid as a fixative. Although this fixative penetrated rapidly it was not satisfactory for fleas because it dissolved fat.

Formalin-acetic-alcohol, more familiarly known as FAA, proved to be the most satisfactory fixative. Material could be left in it almost indefinitely and the alcohol and acetic acid counteract each other with regard to hardening and shrinkage.

Once fixed in FAA, the specimens were then dehydrated through the alcohol series to avoid excessive distortion of the internal organs. At the same time, since the dehydration procedure causes hardening, the interval between changes was finally set at twenty minutes.

Xylene was not satisfactory as a clearing agent. It caused the exoskeleton to become brittle. It also caused distortion and shrinkage of the internal structures. Cedarwood oil was substituted for xylene as a clearing agent because it penetrated rapidly and did not harden the tissues; however, it proved to be very difficult to remove from the specimens when they were put in melted paraffin. The traces of this non-volatile oil that usually remained made sectioning a problem because, in spots, the ribbon was almost "mushy" or fluid.

Benzene, another clearing or dealcoholizing agent, was also tested. It proved to be far superior to xylene as it did not harden the specimens. Chloroform was the most practical in that it is the best solvent of paraffin. However, when it was used, as in the case of using xylene and benzene, the specimens had to be completely dehydrated.

Several embedding media, such as paraffin of varying hardnesses and melting points and celloidin, as well as double embedding were used in repeated attempts to get good serial sections.

The paraffin embedding was convenient in that it was fast and serial sections were obtained with ease. However, as heat is required and it is difficult to determine the minimum amount of heat needed for paraffin infiltration, excessive hardening and shrinkage were a constant problem.

In order to avoid heating and be confronted by the difficulty of further hardening of an already hard (unyielding) exoskeleton of the specimens, I resorted to celloidin. The use of this material was too time consuming and messy. As serial sections were not easily obtained, the procedure was quickly discarded.

Inasmuch as I already had a number of infected fleas being processed in thin celloiden, I attempted double embedding. This procedure gave slightly better results than the paraffin method. The internal structures were better supported by the celloidin, and the paraffin embedding enabled me to serial section the specimens.

When the celloidin-impregnated specimens were heated during the paraffin embedding, the heat not only hardened the chitin but also the celloidin. A second problem developed when the time interval between changes of dehydrating substances in the celloidin process was shortened, resulting in considerable distortion of internal structures.

The slight advantage of obtaining better results by the use of double embedding was offset by two factors. These were (1) hardening of both celloidin and chitin when the paraffin embedding time was lengthened or (2) incomplete paraffin infiltration if the time was shortened.

From the standpoints of time, convenience, and results I felt that the paraffin embedding would be adequate if its melting point were lowered and its firmness increased for thick sectioning. One part of bayberry wax to ten parts of paraffin (tissuematt 58° C.) was used. The resultant embedding wax had a lower melting point (52° C.), and increased firmness. Furthermore, the addition of bayberry wax removed the occasional problem of crystallization in the paraffin when cooling.

Once the bayberry paraffin technique was developed, it was used exclusively in preparing my specimens for sectioning. After being satisfactorily embedded, the fleas were sectioned on a rotary microtome with a new razor blade used for each insect. Sections were cut at 20 microns as only gross appearance of the organs was of importance.

Blood Smears of Dogs

In the early stage of the investigation to find prelarvae I checked the blood of dogs using the technique described by Phillips (1939). This method involves the making of a tiny incision on the inner surface of the ear and then placing a large drop of blood obtained from the cut on a glass slide. A cover slip is then placed on the drop and the blood immediately examined with 440 magnification.

On four occasions autopsies showed adult filariae where the smears were negative. The adult filariae in these four exceptions were examined. In each case both male and female filariae were present. The adult female filariae taken from the dog's heart were immediately cut into segments about eight millimeters long. These parts were then placed in a petri dish containing physiological saline and examined under a dissecting microscope with 120 magnification. As the contents of each segment were removed with dissecting needles, great numbers of active prelarvae were observed. After checking Hinman's (1935) excellent paper on D. immitis periodicity, I concluded that a better technique for sampling blood was necessary as fertile female adult filariae were present and periodicity not too marked.

Several techniques for obtaining blood samples, as described in various laboratory texts, were tried; however, I finally set up my own procedure and this proved very satisfactory.

A minimum of two cc. of blood is withdrawn from a vein

located below the patella of the dog. This vessel is quite prominent after the area has been shaved and can be further accentuated by applying xylene to the skin. A hypodermic syringe equipped with a number 22 gauge needle proved to be the most effective in getting blood samples. The drawn blood is discharged into a small, rubber-capped vial which previously had been rinsed with a solution of heparin. If necessary, these vials can be stored for several days in a refrigerator kept at about 6° C. The contents of the vials are then poured into centrifuge tubes and centrifuged at 3600 RPM for eighty seconds. The supernatant is poured off and the concentrate spread on slides for thick smears and dyed with Wright's stain.

This technique needed refinement in speed and time of centrifuging, in quantity of the sample required, and storage period. While these refinements were being considered, Burch and Blair (1951) published their method which incorporated the refinements originally lacking in my technique. They used two cc. samples of blood centrifuged at 4500 RPM for one minute, and after pouring off the supernatant examined the last drop. This resulted in a more efficient and satisfactory concentration of the prelarvae.

FLEAS AS INTERMEDIATE HOSTS OF *D. IMMITIS* LARVAE

Review of Literature

During the latter part of the 19th century many workers in various countries, particularly Lewis (1875), Manson (1877), Grassi (1888), and Sonsino (1888) tried to find the intermediate host for *D. immitis*. Sonsino (1888) reported finding some *D. immitis* larvae not only in the flea (*Pulex serraticips*), but also in the louse (*Halmatopinus piliferus*). Grassi (1890) and Calandruccio (1890) were not able to confirm Sonsino's observation with regard to finding filarial larvae in the louse; however, they found embryonic nematodes in the fleas *Pulex serraticips* and *P. irritans* and in the tick *Rhipicephalus siculus*. The following year Grassi (1890) changed his opinion. He conceded that fleas and lice ingest the prelarvae. The prelarvae do not undergo metamorphosis in these ectoparasites, but instead die in the intestine of the arthropods. Bancroft (1901, p. 41) examined *Pulex serraticips*, the common stablefly *Stomoxys*, *Culex vigilax*, a mosquito, and the intestinal worm *Ancylostoma*. He concluded:

> All these animals abstract together with blood the embryos, but the latter appear not to enter upon a metamorphosis, and after several days can no longer be traced; it is thought that they are digested.

Breinl (1921) experimented with Ctenocephalides felis and C. canis. He reported that the metamorphosis of the D. immitis larvae took place in the malpighian tubules of the fleas. Phillips (1939, p. 122) stated:

> Early in the investigation 21 fleas, Ctenocephalides canis, taken from the Russian hound /heavily infected/, were dissected and examined for filariae /prelarvae and filaria larvae/. None was infected. From these negative findings it was apparent that fleas were not important intermediate hosts here, and further study of fleas in this connection was not made.

Summers (1940) reported finding both prelarvae and developing larvae in Ctenocephalides felis and C. canis. On no occasion did he find any in the malpighian tubules as reported by Breinl (1921).

Bradley (1952) reported that the cat flea Ctenocephalides felis may be susceptible under certain conditions.

It can be seen, therefore, that there is no concurrence of opinion with regard to the susceptibility of fleas to D. immitis or to their ability to carry the worms to the infective stage.

Incidence of Infection of Fleas with D. immitis Larvae

Fleas were collected from 71 of the 142 dogs examined during this investigation. The 1203 fleas collected were removed from both

infected and non-infected dogs. As I found Ctenocephalides felis to be the most prevalent flea on dogs in central Florida, I concentrated my collecting, separation of sexes, and dissecting on this species. The other genera and species of fleas occasionally found on dogs were collected and colonies maintained in the laboratory for rearing and additional experiments. The species used were as follows: Ctenocephalides canis, Xenopsylla cheopis, Pulex irritans, Echidnophaga gallinacia, and Orchopeas wickhami. The classification is that of Fox (1940)

Of the 342 male Ctenocephalides felis and 861 female C. felis collected and dissected I found advanced D. immitis larval development in 111 males and in 335 females. The preponderance of female fleas collected and subsequently dissected was due to their greater size and ease of collection. Many male fleas were seen on the dogs from which specimens were taken; however, the males proved to be more elusive than the large, egg-laden females. Results of the collections and dissections are summarized in Table 1.

The presence of prelarvae and early larval stages was observed in more than the 446 specimens which showed advanced stages. As I was anxious to establish the fact that the flea is not a refractory intermediate host, in which only limited development took place, I disregarded the earlier stages in recording positive findings in this series of dissections. All the advanced "sausage stage" larvae, pre-infective and infective stage larvae were found in the haemocoele of the abdomen and thorax.

TABLE 1

INCIDENCE OF INFECTION OF D. IMMITIS LARVAE IN CTENOCEPHALIDES FELIS

BREED AND CASE NUMBER OF DOG EXAMINED	CASES FLEAS NOT COLLECTED	NUMBER OF FLEAS DISSECTED			FLEAS WITH ADVANCED DEVELOPMENT OF MICROFILARIAE		
		Male	Female	Total	Male	Female	Total
Shepherds 6, 49, 52, 85 95, 114, 121	2	30	34	64	3	14	17
Collies 3, 5, 73, 104 115, 119, 120	2	25	58	83	5	29	34
Poodles 48, 53, 105, 107	2	8	17	25	..	4	4
Chows 50, 51, 87	1	17	41	58	5	26	31
Cocker Spaniels 20-22, 46, 47 86, 116, 136 140, 141	5	10	25	35	1	10	11
English Setters 4, 34, 35, 118	1	2	148	150	2	12	14
Hounds 1, 2, 18, 29 30-33, 41, 42 72, 74-81, 89-94 100-103, 117 134, 135, 139	6	149	375	524	51	155	206
Bulldogs & Boxers 83, 98, 99, 132 133, 137, 138	..	26	22	48	11	14	25
Fox Terriers 8, 36-38, 44 45, 82, 88, 97 106, 111, 122	6	30	36	66	14	19	33
Mongrels 7, 9-17, 19, 39 40, 43, 54-71 84, 96, 108-110 112, 113,123-131 142	40	45	105	150	19	52	71
Totals	65	342	861	1203	111	335	446
Percent Infection					32%	39%	35.4%

Under natural conditions, I found that thirty-five percent of all the fleas collected were infected with larvae in advanced stages of development. On several occasions I observed fleas moving from one dog to another when the dogs were in close contact. Thus, it appears that fleas, with their high incidence of infection, act as intermediate hosts for the dog heartworm and can readily be spread from one animal to another through direct contact. Fleas may also drop from an infected dog and later attach themselves to another dog. Both methods of transfer of the fleas have been observed.

In order to determine the percentage of infection of fleas feeding on an infected host I set up an experiment in which the fleas were restricted to the back of an infected dog. After fourteen days forty of the fleas were removed, sexed, dissected, and examined for prelarval and larval forms. Thirty-eight of the fleas showed some evidence of infection. Results of the forty dissections are tabulated in Table 2. From this high percentage (95%) of positives can be seen that fleas are very susceptible to infection with D. immitis.

In many cases prelarvae from recent blood meals were present as well as all stages of developing larvae ingested during preceding blood meals. The positions of the various larval stages in the body of the flea are also summarized in Table 2.

TABLE 2

POSITION AND NO. OF LARVAE OF D. IMMITIS IN THE BODY OF FLEAS[a]

FLEAS No.	DISSECTED Sex	Prelarval	1st Stage	2nd Stage	3rd Stage	4th Stage (Infective)
1	female	5 midgut	7 h. a.	8 h. a.	1 h. t.	1 h. t.
2	"	3 midgut	.....	3 h. a.	.....	1 mouthparts
3	"	5 midgut	.....	.....	3 h. a.	2 h. t.
4	male	39 midgut	.....	.....	.....	.....
5	female	....	.....	.....	.....	.....
6	"	13 midgut	5 h. a.	.....	.....	.....
7	"	7 midgut	4 h. a.	.....	1 h. t.	.....
8	male	4 midgut	.....	1 h. a.	.....	.....
9	"	7 midgut	1 h. a.	2 h. a.	1 h. a.	2 h. t.
10	female	3 midgut	2 h. a.	.....	.....	1 h. t.

[a]All fleas dissected were Ctenocephalides felis with the exceptions of Nos. 12, 13, 14, 37, 39 and 40 which were C. canis.

[b]h. a. - haemocoele of abdomen
h. t. - haemocoele of thorax
h. h. - haemocoele of head

TABLE 2--Continued

FLEAS No.	DISSECTED Sex	Prelarval	1st Stage	2nd Stage	3rd Stage	4th Stage (Infective)
11	female	11 midgut	.....	1 h. a.	2 h. t.	.....
12	male	6 midgut	.....	.....	.....	.....
13	female	12 midgut	.....	1 h. a.	.....	.....
14	"	1 midgut	3 h. a.	.....	1 h. a.	.....
15	"	....	.....	1 h. a.	.....	.....
16	male	5 midgut	.....	.....	.....	.....
17	female	2 midgut	.....	1 h. a.	.....	.....
18	"	5 midgut	3 h. a.	2 h. a.	.....	.....
19	"	....	.....	2 h. a.	.....	1 h. a.
20	male	11 midgut	8 h. a.	.....	.....	.....
21	"	1 midgut	.....	.....	.....	.....
22	male	3 midgut	.....	1 h. a.	.....	.....

TABLE 2--Continued

FLEAS No.	DISSECTED Sex	Prelarval	1st Stage	2nd Stage	3rd Stage	4th Stage (Infective)
23	male	2 midgut	5 h. a.	1 h. a.	1 h. t.	.....
24	"	....	.....	6 h. a.	2 h. t.	.....
25	female	5 midgut	2 h. a.	8 h. a.	1 h. t.	1 h. t.
26	"	....	.....	.....	.....	1 out of mouthpart
27	"	....	.....	1 h. a.	1 h. t.	.....
28	"	4 midgut	2 h. a.	.....	.....	.....
29	"	3 midgut	8 h. a.	4 h. a.	2 h. t.	3 h. a. (1) h. t. (2)
30	"	....	.....	.....	.....	.....
31	"	....	2 h. a.	7 h. a.	1 h. t.	.....
32	male	2 h. a.	11 h. t.	1 h. a.	2 h. a.	.....
33	"	6 h. a.	.....	.....	.....	4 h. a. (3) h. t. (1)

TABLE 2--Continued

FLEAS No.	DISSECTED Sex	Prelarval	1st Stage	2nd Stage	3rd Stage	4th Stage (Infective)
34	male	1 midgut	.....	.....	.....	.....
35	female	....	.....	4 h. a.	3 h. a.	2 h. h. or h. t.
36	"	....	.....	4 h. a.	3 h. a.	2 h. h. or h. t.
37	male	17 midgut	6 h. a.	.....	4 h. t.	.....
38	female	11 midgut	6 h. a.	7 h. a.	.....	.....
39	"	2 midgut	.....	.....	.....	.....
40	"	31 midgut	.....	.....	8 h. t.	5 h. h. or h. t.

METAMORPHOSIS OF LARVA OF D. IMMITIS

Method of Study

It was shown earlier in this investigation that thirty-five percent of the fleas collected from dogs were infected with larvae of D. immitis in advanced stages of development.

A number of experiments were conducted so that the time required for the larval metamorphosis from the prelarval form of the prelarva to the infective stage could be determined. To do this, series of laboratory-reared, infection-free adults of Ctenocephalides felis, C. canis, Xenopsylla cheopis, Echidnophaga gallinacea, Pulex irritans, and several Orchopeas wickhami were allowed to feed on an infected dog that, previous to the experiment, had been cleared of all ectoparasites.

During the feeding the fleas were restricted in large test tubes and fed through a fine bronze screen (40 x 40 mesh per inch). The host dog was tied back down on a rabbit board and given a mild anesthesia. Under these conditions it was possible to have the fleas feed for extended periods of time on the shaved abdomen of the dog.

It was a simple matter to note when the fleas were replete by the distention of the abdomen of those which had fed. When no further

feeding was taking place, the fleas were placed in gallon jars which contained a sheet of paper toweling to absorb excretory wastes. The jars were capped with fine gauze to prevent the fleas from escaping and stored in a constant temperature and humidity cabinet.

Young rats in restraining cages were placed in some of these jars as a source for continued feeding. A dissection schedule was then set up for all of the fleas that had fed on the definitive host.

During the first six hours after taking a blood meal, samples were taken of all genera and species of fleas involved as well as of both sexes, and these were dissected at thirty minute intervals. During the following six hours specimens were dissected at one hour intervals. Thereafter the remaining individuals were dissected at twelve hour intervals.

More than 1100 laboratory-reared fleas were dissected at set time intervals after they had fed on an infected host. In this way I was able to plot the time, form of the developing larva, and its location in the intermediate host.

Dissections carried out during the desired time intervals required the use of twelve fleas (six species with a male and female of each). As there were 32 scheduled periods, a total of 384 fleas was required. When the first of three sets of dissections was made, more than the required 384 fleas were available and so were used. In repeating the experiment for the second and third times as a check against the initial findings, only the more prevalent species

(*Ctenocephalides felis*, *C. canis*, and *Xenopsylla cheopis*) were reared in sufficient numbers for all of the scheduled dissections.

Dissection Procedure

A flea that had fed on the infected, definitive host was lightly anesthetized by ether and placed in a drop of physiological saline solution on a slide. The suspected intermediate host was then dissected with a series of minute scalpels fashioned from No. 4 insect pins that had been heat tempered and finely honed.

In fleas suspected of harboring advanced stages of larval development, the mouthparts were palpated and examined prior to head removal. Next the head was severed where it joined the prothorax. The severed head was then moved aside in the saline. The ventral surface of the thorax and abdomen were opened and checked for larval forms in the haemocoele. The next parts removed were the reproductive organs after they had been carefully separated and examined. Finally, the complete alimentary tract was dissected out, less a short section of the esophagus and the entire pharynx which were attached in the severed head.

The alimentary tract was carefully examined for perforations to check whether or not the dissection was successful in preventing easy exit from the midgut into the haemocoele for some of the weaker prelarval forms.

The primary dissections such as decapitation and opening the

abdomen were performed under a magnification of 67.5. The dissections of the organ systems were made with a magnification of 112.

Description of the Metamorphosis of the Larva of D. immitis

The metamorphosis of D. immitis prelarva in Ctenocephalides felis, C. canis, Xenopsylla cheopis, Pulex irritans, Echidnophaga gallinacea, and Orchopeas wickhami at 27° to 29° C. shows the typical morphological changes that occur in the development of all filarial larvae as described by Connal and Connal (1922) for Loa loa larvae in Chrysops silacea, Blacklock (1926) for Onchocerca volvulus larvae in Simulium damnosum, and Yamada (1927) for Wuchereria bancrofti larvae in Culex pipiens.

The prelarva of D. immitis is a sheathless form. In the course of my dissections, I found that some of these prelarval forms had penetrated the midgut and entered the ventral region of the abdominal haemocoele as early as one hour after having been ingested with a blood meal by the flea (Figure 1).

Fleas dissected two, three, four, and five hours after a blood meal showed a progressive increase in the number of prelarval forms located in the haemocoele in contrast to the numbers still active and free in the midgut.

After having already dissected more than 300 infected fleas, the earliest penetration of prelarvae into the haemocoele of the abdomen which I observed was less than one hour after the worm was ingested.

However, the greatest number penetrated within four to five hours after being taken in with the blood meal. On the basis of this finding I concluded that in the remaining 264 required dissections to complete my experiment, all larval forms would be found outside of the midgut.

Later in the course of the experiment I found several exceptions to this conclusion. In one case an active prelarva was still present in the midgut of a female Ctenocephalides felis thirty-six hours after having been ingested. This appears to be a purely accidental exception. Once in the haemocoele the prelarval form undergoes distinct changes in structure. During the first six hours in the abdominal cavity the internal structures of the prelarval form, such as the nerve ring, excretory pore, and genital cells become more obvious (Figure 2).

The following twenty-four hours are the most striking in that the developing larva appears to be compressed to two-fifths of its prelarval length and at the same time doubles its diameter. This phase of development varied in the different intermediate hosts; however, in all cases the larvae underwent thickening and shortening and developed a thin, short tail. These first-stage, stumpy larvae are 90 to 105 microns in length and about 16 microns in diameter and are found throughout the entire abdominal and thoracic haemocoele (Figure 3).

During the second stage of development, the larva continues to thicken in diameter and starts elongating. The majority of these

characteristic sausage forms measure about 240 microns in length and 30 microns in diameter. The thin tail present on these forms is no longer extended as earlier but instead shortens and is bent like a hook. The esophagus, intestine, and anus are very distinct in these massive larvae. The larvae during this stage are sluggish and are all localized in the ventral region of the abdominal haemocoele. The dissections showed that the prelarvae develop into this characteristic massive "sausage stage" as early as forty-eight hours after having been ingested by the flea. Seventy-two hours after the blood meal on the infected host, only an occasional "sausage stage" larva was observed in the infected fleas (Figure 4).

After seventy-two hours the third-stage larva was frequently found. This form, occurring throughout the entire body of the flea, is very active in contrast with the sluggish form from which it developed. In addition to its increase in length to 400 microns to 600 microns, the buccal cavity of the larva is more distinct, and the esophagus is now thin, while the posterior region remains a broad mass. Some of these early third-stage larvae still retain a reduced, hook-like tail (Figure 5).

One hundred fifty-six hours after the initial blood meal, I began finding mature infective stage larvae which measured 1600 microns in length and more than 20 microns in diameter. After 180 hours almost all the larvae in the fleas were infective.

The majority of the infective stage larvae were located in

the relatively uncrowded haemocoele of the head and thorax. However, a number were found lodged among the reproductive and excretory organs; these dislodged themselves and moved actively in the saline when the viscera were removed from the crowded abdomen (Figure 6).

My observations on the development of D. immitis and its movements in the body of the flea are summarized in Table 3.

Aberrant Behavior

One of the female Ctenocephalides felis, dissected thirty-six hours after the blood meal, had an active prelarva in the midgut. At the same time several early-stage, larval forms were observed in the abdominal haemocoele. Apparently this prelarval form was an old prelarva which lacked the vigor of the others ingested along with it and was unable to penetrate the gut wall. Underwood and Harwood (1938) reported maximum life span of two years, four and one-half months, for the prelarva of D. immitis in the circulating blood of the dog. There appears to be no information relative to the length of life span of prelarvae in the gut of insects.

During another dissection, as the anesthetized female flea (Ctenocephalides felis) was being placed in a drop of physiological saline on a slide, the flea defecated some of the blood meal. Two active prelarvae were among the partially digested red blood cells. This suggests that fleas may normally excrete many of the ingested prelarvae before they have an opportunity of passing through the gut wall.

TABLE 3

DEVELOPMENT OF D. IMMITIS
LARVA IN FLEAS[a]

Time	Form of Larva	Location in Flea
Blood meal plus		
30 min.		midgut
1 hr.		
1½ hrs.		
2 hrs.		invading the stomach wall[b]
2½ hrs.	distinct prelarval stages	
3 hrs.		
3½ hrs.		in ventral abdominal haemocoele
4 hrs.		
4½ hrs.		
5 hrs.		
5½ hrs.	internal structures becoming more distinct during transition from prelarval to early larval stage	throughout the haemocoele of thorax and abdomen
6 hrs.		
7 hrs.		
8 hrs.		
9 hrs.		
10 hrs.		
11 hrs.		
12 hrs.	first larval stage with a distinct, thin tail	
24 hrs.		
36 hrs.		
48 hrs.	characteristic sausage form of 2nd stage larva	haemocoele of abdomen
60 hrs.		
72 hrs.	preinfective form 3rd stage larva	haemocoele of abdomen
84 hrs.		
96 hrs.		
108 hrs.		
120 hrs.		
132 hrs.		
144 hrs.		
156 hrs.	mature infective stage larva	majority in the haemocoele of the head and thorax. Some in abdominal haemocoele.
168 hrs.		
180 hrs.		

[a] Ctenocephalides felis, C. canis, Xenopsylla cheopis, Pulex irritans, Echidnophaga gallinacea, Orchopeas wickhami.

[b] Dotted red line indicates time of penetration of stomach wall by prelarval forms.

EFFECTS OF *D. IMMITIS* LARVAE ON FLEAS

As was reported earlier (Page 3), mosquitoes that are susceptible have a high mortality rate when they are infected with *D. immitis* larvae. Unlike the larvae of other filariae such as *Wuchereria bancrofti* and *W. malayi*, which metamorphose among the thoracic muscles of the mosquito, the larvae of *D. immitis* undergoes the early stages of development in the malpighian tubules of the mosquito. In the course of this development the excretory organs of the mosquito are subjected to extensive damage so that it is improbable that they are still functional. Mortality rates as high as 100 percent due to this damage were recorded by Bradley (1953) for some species of mosquitoes. In fleas, however, the early development of *D. immitis* larvae occurs in the haemocoele rather than in an essential organ.

In order to be able to approximate the mortality rate of infected fleas as compared to infection-free fleas, I used eleven colonies of fleas separated into three experimental groups. Groups A and B were the colonies of infected fleas and Group C composed of three infection-free colonies. Group A consisted of six colonies of laboratory-reared, infection-free fleas. They took their first blood

meal on an infected dog. Subsequent feedings by Group A were made on young rats confined in small cages to restrict movement. Group B consisted of two colonies of fleas taken from infected dogs. These were placed on young rats for further feedings. Group C, made up of three laboratory-reared, infection-free colonies used as a control, fed exclusively on young rats. In each of the eleven colonies composing Groups A, B, and C twelve male and twelve female fleas were kept on ectoparasite-free, young rats.

Observations on anesthetized fleas and sample dissections of other fleas from Groups A and B were made and their infections verified by discovering the presence of developing larvae of D. immitis.

Daily observations made over a twenty-day period showed no difference as to mortality rate of the infected fleas of Groups A and B as compared to the infection-free fleas of my control Group C.

I noted the presence of flea eggs in as great numbers in Groups A and B as in Group C. Counts of flea eggs present in all the eleven colonies were not made because of two factors: (1) the question of viability of the eggs, and (2) the time required for an accurate count of the enormous number produced. The problem that was presented was solved rather simply. I merely waited until the eggs hatched and the flea larvae went into the pupal stage. After sifting the pupae out of the sand and debris in the rearing cages, I measured their volumes in a graduate cylinder. The volumes of pupae of the control Group C were not larger than the volumes of pupae which were the

offspring of the infected colonies comprising Groups A and B. The pupae of all colonies were kept for adult emergence; here again there was no apparent difference as to numbers of adults.

The results of these experiments clearly show that there is no increase in the mortality rate nor apparent effect on the fecundity of the female fleas or fertility of the male fleas harboring an infection of D. immitis larvae.

BEHAVIOR OF D. IMMITIS LARVAE

Activity of Larvae in the Flea

As was mentioned earlier in the discussion of larval metamorphosis (Pages 27 and 28), the majority of the infective stage larvae were found in the haemocoele of the head and thorax of the dissected infected fleas. These infective stage larvae, because of their high degree of motility, were able to move against the haemolymph flow and leave the crowded, organ-packed haemocoele of the abdomen.

In order to observe the movements of the infective stage larvae in the living flea, I tried several restraining media such as polyvinyl alcohol and methyl cellulose. These were not effective in restricting the movements of the active flea; therefore, I employed ether to anesthetize the insects.

The lightly anesthetized flea was then placed on a glass slide and the internal regions of the head, thorax, and abdomen were observed with the aid of a 100 and 440 magnification. In each case a strong beam of transmitted light rather than reflected light was used as a light source.

In almost all cases the activity of the worm larvae could be

observed in the flea. The greatest difficulty was encountered when some of the larvae moved into the abdominal haemocoele. The crowded organs were seen being pushed about in the lightly chitinized Xenopsylla cheopis as the active larvae moved among them. The larvae were visible at times; however, when they stopped their movement it was impossible to distinguish them from the internal organs of the flea.

The movement of the larvae in the haemocoele of the thorax and abdomen can best be described as being that of a typical nematode. In the haemocoele of the head, however, they displayed more of a gliding action with a minimum of lateral motion. I began to speculate as to why the activity in the haemocoele of the head was such a contrast to the rather violent motion exhibited in the thorax and abdomen. Then I noticed that one of the larvae that had apparently penetrated, in part, the minute haemocoele of the labrum suddenly move backwards. The gliding movement exhibited by the larva in the haemocoele of the head was the result of the restricted area for movement.

I was able to induce the backward movement of the larva from the head into the thorax by gently applying an ice cold blunt dissecting pin to the mouthparts of the flea. In other tests, I put a drop of saline over the mouthparts of the anesthetized flea and shortly thereafter could observe the forward movement of the larvae wriggling in the abdomen, subdued activity in the thorax, and then the

restricted movement into the head.

Method of Escape of the Larva from the Flea

The mechanism of the escape of the infective stage larva from the intermediate to the definitive host follows a very intricate pattern. The first requirement is that the fully developed larva be located in the vicinity of the mouthparts.

Menon and Ramamurti (1941), in their investigation of Wuchereria bancrofti larvae, found that with moderate warmth (about 35° C.) the infective larvae became very active and could be observed moving to the very tip of the proboscis of infected Culex fatigans. They suggested that warmth was a definite factor in larval escape since it brought about increased activity.

My experiment with the infective stage larvae of D. immitis in various infected fleas also showed a similar pattern of larval concentration in the head region when warm physiological saline was placed on the mouthparts of the flea.

After having succeeded in concentrating the larvae in the haemocoele of the head, I tried a series of stimuli to induce them to leave the body by moving out of the mouthparts. My first test involved use of hot saline (45° - 52° C.) around the mouthparts. With this, those that did not die in place rapidly moved back into the haemocoele of the thorax and abdomen. Cool saline (22° - 24° C.) did not kill any of the larvae; however, all left the near vicinity of the

head and withdrew into the body.

Warm lymph and later warm blood increased the activity of the larvae in that they moved from the haemocoele of the abdomen and thorax into the haemocoele of the head. However, this increased activity was no greater than that brought about by using the warm saline. The results of the test with cold lymph and cold blood were the same as those with the cold saline.

These simple tests showed that the chemical stimulus was incidental. The primary stimulus proved to be the presence of a warm fluid over the mouthparts, for this alone increased the concentration and activity of the larvae in the haemocoele of the head. No escape of the infective stage larva was seen after the external application of warm fluids to the mouthparts.

I realized that I could not duplicate the position of the mouthparts of the anesthetized flea to that of a flea actively feeding. Thereupon I tried to fix an infected flea in the act of feeding on a host. My plans were to serial section the feeding flea and the excised skin of the host, and thus be able to determine the way infective D. immitis larvae enter the definitive host.

The standard fixatives such as Bouin's, the alcohols, formalin combinations, Lo Bianco's fluid, and Gilson's fluid all acted too slowly in that the feeding flea released its mouthparts immediately. The anesthesias such as the ethers, chloroform, ethyl chloride, and carbon dioxide were not satisfactory as the flea shifted almost

immediately after exposure to these substances. The commercial insecticides were all too slow. Contact freezing with dry ice proved to be too awkward. As a last resort I held a shell vial that was partially filled with cotton saturated with a four percent osmic acid solution over a feeding flea. Contrary to all expectations, the flea was still alive ten minutes later! Needless to say, this experiment was abandoned.

The flea, in preparation for taking a blood meal, first makes a preliminary investigation of the skin with the maxillary palps. Then the head is applied to the skin, the abdomen being held at an angle of about 45°. The labial palps are widely spread and the mandibles, the only piercing appendages, are thrust into the skin. The resultant would is caused by the protraction and retraction movement of the madibles. Saliva is poured into the would during this action. The skin of the animal being bitten, due to the saliva of the flea, is, at times, severely irritated. The blood withdrawn from the host moves up the food channel into the pharynx, down the esophagus, through the proventriculus, into the midgut. The distention of the midgut filling with the blood meal from the host brings about a rise in the intra-coelomic pressure of the flea. This rise of the intra-coelomic pressure stretches the intersegmental membranes connecting the exoskeleton and thus may sometimes permit larval escape through the body wall.

I assumed that the easiest location for exit of the larvae would

be in the region of the mouthparts. The labrum-epipharynx, which has been described as the labrum and also the epipharynx by Patton (1913), is a long, slender organ, flattened on its ventral surface where it is in opposition to the mandibles (Plate X). The dorsal wall, corresponding to the epipharynx, are composed of rigid chitin, whereas the lateral walls which connect these structures are membranous. Within these walls is a space continuous with the haemocoele of the head. At times I had observed larvae in the vicinity of this structure. The labium (proboscis) of the flea is the most posterior of the mouthparts. It consists of a short, median, basal, hollowed-out, thin, chitinous plate with two, four-segmented, labial palps, or labella, at its distal end. As mentioned earlier, the labial palps do not enter the wound, but are bent to the right and left as the mandibles enter the skin.

As with the labrum, I observed infective stage larvae in the vicinity of the labium and what appeared to be a partial entry into its haemocoele. During the course of this phase of the investigation I had 14 cases where palpation of the mouthparts, while immersed in warm physiological saline with pressure applied at the same time to the distended abdomen, resulted in the emergence of infective stage larvae from mouthparts of the flea. I was unable to determine which of these short structures was the principal exit point.

On many other occasions when an engorged flea containing infective stage larvae was injured in any way by improper handling, it

was not unusual to have the infective stage larvae emerge by way of the injured areas.

Summers (1943, p. 177) reported that in Pulex irritans

> mature larvae /D. immitis/ were seen penetrating through membranes which connect the chitinous plates composing the body wall of the flea. This was noted especially in the region between the thoracic and abdominal segments.

Penetration of the Host by the Larva

Many of the present day texts have statements like this:

> They /infective larvae of filaria/ are not directly inoculated by the bite /referring to a blood feeding arthropod/ but pierce the skin, enter the circulation... (Stitt, Clough and Clough (1945, p. 513)

I assumed such statements, as the quotation above, to be true, but when I examined serial sections of skin which had been pierced by arthropods infected with several infective stage larvae, my findings were meager. Serial sections made of skin upon which an injured flea that had infective stage larvae emerging from it showed no evidence of larval penetration.

I realized that I knew nothing as to the speed of penetration and where the larvae moved once they had entered the definitive host. Therefore, I decided to try to count the number of infective stage larvae in the flea before and after a blood meal. This, of course, was not accurate. However, by gross examination, as previously described, I was able to estimate whether or not one or more infective

stage larvae were present. In this experiment I used only fleas in which infective stage larvae could be seen. The skin of a new-born rat, in the region of the bite made by the intermediate host, was removed, fixed, sectioned, stained, and checked for the presence and number of infective larvae. The flea that had taken the blood meal was dissected in physiological saline and examined for the presence and number of infective stage larvae.

The majority of the skin samples that were serially sectioned did not show the presence of the larvae. However, an occasional portion of a penetrating larva was found in few samples of skin. At the same time, the infected fleas, both the injured from which larvae were observed exiting onto the skin of the host and the uninjured which I assumed could discharge larvae through the mouthparts, showed a frequent decrease or complete absence of all previously observed infective stage larvae.

In the course of this experiment I observed forty-seven infective stage larvae in twenty-eight infected fleas; the number of larvae was, in all probabilities, higher. The skin samples gave three positives, and in the dissections of the twenty-eight fleas I found seventeen remaining larvae.

Probably more than the twenty-seven infective stage larvae were not accounted for. I began to doubt the statement that larvae penetrate the skin and therefore set up a series of experiments in order to determine whether this was true or not.

A series of infected fleas, Ctenocephalides felis, C. canis Xenopsylla cheopis, and a mosquito, Psorphora ferox, were dissected in saline as a source for infective stage larvae. The larvae were then recovered in micro-pipettes and placed on various types of intact skin in a drop of saline or lymph and observed through a dissecting microscope. The results of this experiment are summarized in Table 4.

The results of placing larvae on intact skin showed that they were incapable of penetrating it. The larvae moved about actively in the droplet of saline. When their moist medium began to evaporate, their movements became more restricted. By the time the drop had completely evaporated, the larvae dried up and died.

The next test used skin that had been broken. I employed insect pins, dissecting needles, and a sharp pointed scalpel to simulate the punctures that an actively feeding flea makes. I realized that the openings lacked the anticoagulin that the feeding flea pours into the wound with its saliva. I tried heparin (1:9000) and also saline; however, this added to the difficulty of temperature regulation, dropping it to such a point that the larvae, sensitive to slight temperature changes and positively thermotropic, would not move toward the wound. Because of these factors and repeated failures, the addition of diluting agents and anticoagulants was discontinued. When the larvae were dropped over the puncture in the skin, penetration into it was rapid. The larvae that were near the

TABLE 4

TESTS SHOWING INABILITY OF LARVA
TO PENETRATE INTACT SKIN

No. of Larvae	Intact Skin	Time Interval	Results
6	Shaved leg of a dog	1 - 15 min.	no penetration, died*
9	Back of a young rat	1 - 15 min.	" " "
3	Belly of a young rat	1 - 15 min.	" " "
4	Ear of a dog	1 - 15 min.	" " "
2	Finger of a man	1 - 15 min.	" " "

*Died because of desiccation.

edges of the puncture merely wriggled back and forth until they dried up.

I believe that when the infective stage larvae escape from the feeding flea, whether through the mouthparts or through the broken body wall caused by the crushing of the flea by the host, they will penetrate the host under these conditions:

1. If they emerge from the flea into a moist medium that prevents their dehydration.

2. If there is warm blood and tissue fluid exuding from the wound (because of the injected saliva containing an anticoagulant).

3. If the distance between the location of the larvae and the wound is bridged with a moist medium.

4. If the distance between the larva and the wound is very short--less than two millimeters.

Breinl (1921, p. 391) stated:

> The mode of transmission was considered, and it was thought possible that under normal conditions the flea would be crushed, and the actively motile larvae thus freed would penetrate through the unbroken skin and reach the blood stream in analogy with the mechanism of Ancylostoma infection.
>
> In order to ascertain the correctness of the surmise, fleas were crushed over the shaved skin of young puppies which had been moistened with saline, and after an interval of from five to twenty minutes the part of the skin was excised and serial sections prepared for microscopical examination
>
> In one instance only out of seven experiments one mature larva was discovered in three consecutive serial sections with the anterior part (about 30 microns of the larva) embedded in

> the subcutaneous tissue, the remaining part adhering to the outer skin. The larva had, without doubt, penetrated through the unbroken skin.

Considering the fact that the exoskeleton of some fleas is thick and brittle, I believe that when these were impressed on the tender skin it produced some breaks in the shaved skin and this gave partial entry for one larva.

INCIDENCE OF INFECTION OF D. IMMITIS IN DOGS

Establishing Incidence of Infection

One hundred and forty-two dogs were examined during this investigation. They ranged from approximately four months through twelve years of age. When a dog was examined, it was given a case number and the following data were recorded on work sheets:

1. The breed of the dog.
2. Whether it was long-haired or short-haired.
3. Age (actual or approximate).
4. Results of blood smear made from blood sample taken.
5. Results of the autopsy if the dog was destroyed for reasons mentioned earlier (Page II).
6. Results of the number of fleas that had been sexed and dissected (Table 1).

Items 1 - 5 are summarized in Table 5.

Of the 142 dogs examined, 89 had positive blood smears showing the presence of prelarvae. Since the time of development of the infective stage larva to the adult filaria was estimated by Hall, Price and Wright (1934) as being a little over eight months, all dogs less than eight months of age theoretically should have been subtracted from the total number of dogs examined for prelarvae of D. immitis.

TABLE 5

INCIDENCE OF INFECTION OF D. IMMITIS IN DOGS

BREED OF DOG	HAIR Long	HAIR Short	AGE Years	BLOOD SMEAR Neg.	BLOOD SMEAR Pos.	AUTOPSY Neg.	AUTOPSY Pos.	AUTOPSY NOT PERFORMED
Shepherds	7		3-5		7	3	3	1
Collies	7		1-2		7	1	3	3
Poodles	4		3-12		4		3	1
Chows	3		3-5		3		3	
Cocker Spaniels	10		1-5	3	7	2	1	7
English Setters	4		7-10		4		1	3
Hounds		33	1½-9	4	29	4	18	11
Bulldogs and Boxers		7	2		7		3	4
Fox Terriers		12	10 mo-9 yrs	5	7	2	7	3
Wire and Sky Terriers	6		7 mo-1½ yrs	5	1			6
Mongrels	5	44	4 mo-9 yrs	36	13	9	12	28
Totals	46	96		53	89	21	54	67
Percent Infection	79	54						

The percentage of the incidence of infection would rise from 63 percent to 77 percent if the 28 dogs less than eight months of age were subtracted from the total number examined. However, I included these dogs because two of them, known to be less than seven months of age, were found to have prelarvae in their blood samples. Furthermore, Augustine (1938, p. 393) reported two five months old cocker spaniel puppies as being infected. He stated:

> The examination of the mother's blood was negative for microfilariae. If prenatal infection did not occur, the absence of the infection in the mother may indicate a decided preference of the infective stage of the parasite from the mosquito for fetal tissue, such as is known to be true of the infective stages of an ascarid (Toxocara canis) and hookworm (Ancylostoma caninum) in prenatal infections in dogs. (Augustine, 1927; Foster, 1932).

I performed autopsies on 75 of the 142 dogs examined. The autopsies were performed on dogs that had been destroyed because of extensive injuries or disease. The following organs, which might be involved, were examined in this sequence for adult filariae.

1. the four chambers of the heart
2. postcaval vein (inferior vena cava)
3. pulmonary arteries
4. lungs
5. thoracic cavity
6. diaphragm
7. mesentery

The adult filariae of D. immitis were usually found in both the right auricle and right ventricle. In severe cases an appreciable number were found in the pulmonary artery, and some in the lungs. The majority of the autopsies revealed about twelve to eighteen present.

In one case I found 109 adult filariae in the heart and thirteen in the pulmonary arteries. Megnin (1883) reported several hundred adult filariae present in the heart of one infected dog.

The worms, when present in great numbers, were interlaced in an almost inextricable way into huge masses which, to some extent, obstructed the cavities of the heart. The filariae were partially wound around the chordae tendinae for attachment, and unattached ends of their bodies hung in the lumen of the heart.

In fifty-four autopsies I found adult filariae; however, in four of these dogs the blood smears showed no evidence of prelarvae. The adult male and female filariae were present in each of these four exceptions. Dissection of the female filariae revealed that they were fertile. Fortunately these autopsies were performed during the early stage of my investigation and emphasized the need of a more accurate method of preparing blood smears. The method of dissection of the filariae and preparation of blood smears are described on Page 11.

Later in the study examples were found in which the blood smears were positive but no adult filariae were found. As stated earlier (Page 28), Underwood and Harwood (1939) reported the maximum life span of the microfilariae of *D. immitis* as being two years four and one-half months in the circulating blood of an infected dog. When eight cases of infection showed the presence of prelarvae and a thorough autopsy revealed no filariae, I assumed that they had died

and subsequently fragmented. Leidy (1881), on two occasions, witnessed the actual vomitting out of living filariae. These filariae probably had been dislodged from the right side of the heart and were transported through the pulmonary arteries to the lungs from which they were discharged after severe fits of coughing.

Comparison of Long-haired and Short-haired Dogs with Respect to Incidence of Infection

Of the dogs examined in the course of this investigation, 46 were long-haired and 96 short-haired. These 142 dogs represented twelve distinct breeds while 49 were non-descript mongrels.

Upon examination of blood smears, I discovered that, of the 46 long-haired dogs examined, 37 of them (79 percent) were positive with prelarvae present. Among the 96 short-haired dogs I found 52 dogs (54 percent) infected. Autopsy findings on specific cases showed no difference between long-haired and short-haired dogs as to the intensity of adult filarial infection. These findings were not in agreement with thos of Hall, Price and Wright (1934) as mentioned on Page 6.

The breed of the dog appears to have no influence whatsoever on susceptibility to D. immitis. This conclusion is based on the study of ninety-three dogs which fell into twelve distinct breeds showing marked resistance to infection.

In the cases where autopsies were performed, again there was no visible difference as to the intensity of adult filarial infection.

The small number of mongrels infected is not an indication of resistance to D. immitis, but rather due to the fact that twenty-six of the number examined were puppies between the ages of four to ten months. Their blood smears were all negative probably because they had not been exposed to prenatal infection.

SUMMARY AND CONCLUSIONS

A study of the relationships existing between the dog heartworm, Dirofilaria immitis, and various species of fleas was undertaken in order to determine the effects of the parasite on a potential intermediate host. The findings and conclusions resulting from this study are summarized below:

1. Thirty-five percent of 1203 Ctenocephalides felis, collected from seventy-one dogs were found to be infected with D. immitis larvae in advanced stages of development.

2. When specimens of Ctenocephalides felis and C. canis were restricted to feeding on an infected dog, ninety-eight percent of them became infected with D. immitis.

3. The development of D. immitis microfilariae to the infective stage was traced in both males and females of Ctenocephalides felis, C. canis, Xenopsylla cheopis, Pulex irritans, Echidnophaga gallinacea, and Orchopeas wickhami.

4. The prelarval forms of D. immitis penetrated the midgut wall and entered the abdominal haemocoele of the flea as early as one hour after having been ingested with a blood meal.

5. The first and second stages of development of D. immitis

larvae take place in the haemocoele of the abdomen.

6. The third-stage larvae were found in the haemocoele of the abdomen and thorax.

7. The majority of the infective stage larvae were found in the haemocoele of the head and thorax.

8. Fleas may egest microfilariae when partially digested blood meals are defecated.

9. Dissections and serial sections of infected fleas showed that no major tissue damage or apparent injury of any organs could be attributed to the developing larvae.

10. The fertility of the male fleas was not affected in any way by the developing larvae, nor was the egg production of the female fleas changed by the larvae infecting them.

11. There was no apparent increase in the mortality rate of infected fleas as compared to the mortality rate of non-infected fleas.

12. The infective stage larvae were shown to be thermotropic concentrating in the haemocoele of the head of the flea when warm saline was placed on its mouthparts.

13. The infective stage larvae are apparently incapable of moving out of an unfed flea.

14. In fourteen cases infective stage larvae escaped through the region of the mouthparts of fed fleas when the intracoelomic pressure of the flea was increased.

15. Infective stage larvae were observed leaving the body of

the flea through injured areas of its exoskeleton.

16. Infective stage larvae proved to be incapable of penetrating intact skin.

17. Prior to feeding, approximately forty-seven infective stage larvae were observed in twenty-eight infected fleas. After feeding, the fleas were dissected and revealed only seventeen remaining infective stage larvae. Skin samples taken from the areas bitten by the fleas showed the presence of three D. immitis larvae in the punctures caused by the feeding of the fleas. This demonstrates that infective stage larvae are capable of leaving the flea and entering the skin puncture made by it.

18. Sixty-three percent of 142 dogs examined had positive blood smears showing the presence of microfilariae of D. immitis.

19. There was no evidence in dogs of breed resistance to D. immitis, nor was the intensity of infection dependent on whether a dog was long-haired or short-haired.

20. Because of the high percentage of infected fleas found under natural conditions, and because of the close association between dogs and fleas, these insects are most likely the natural vectors of canine filariasis.

PLATES AND FIGURES

PLATE I

FIGURE 1

FIGURE 2

Figure 1. Prelarva of Dirofilaria immitis (270 microns long and 6 microns diameter).

Figure 2. Start of first molt of larva (270 microns long and 6 microns diameter).

Abbreviations

AP, anal pore; EC, excretory cell; EP, excretory pore; G1, G2, G3, G4, genital cells; NR, nerve ring.

PLATE II

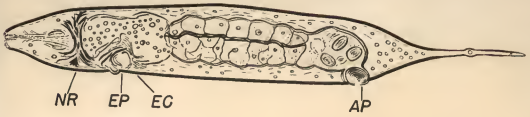


FIGURE 3

FIGURE 4

Figure 3. Early sausage stage larva (105 microns long and 16 microns diameter).

Figure 4. Second stage larva, a distinct sausage stage (240 microns long and 30 microns diameter).

Abbreviations

A, anus; EC, excretory cell; EP, excretory pore; NR, nerve ring; R, rectum.

PLATE III

FIGURE 5

FIGURE 6

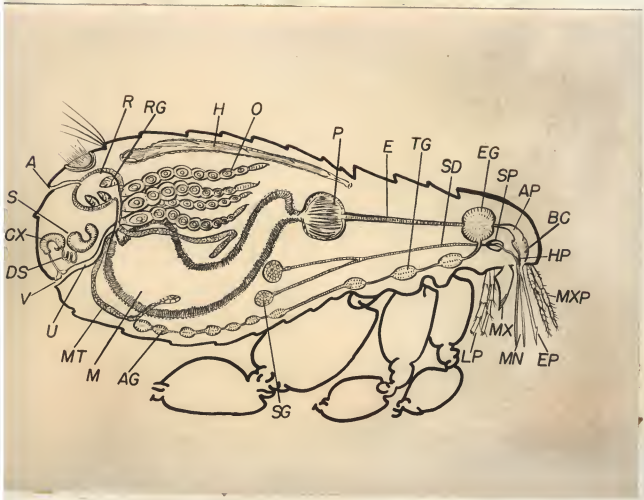
Figure 5. Third stage larva, entering second molt (600 microns long and about 20 microns diameter).

Figure 6. Infective stage larva (1600 microns long and 20 microns diameter).

Abbreviations

A, anus; EC, excretory cell; EP, excretory pore; GA, genital anlage; NR, nerve ring; R, rectum.

PLATE IV

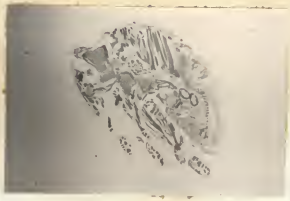


Internal Anatomy of a Flea (sagittal section)

Abbreviations

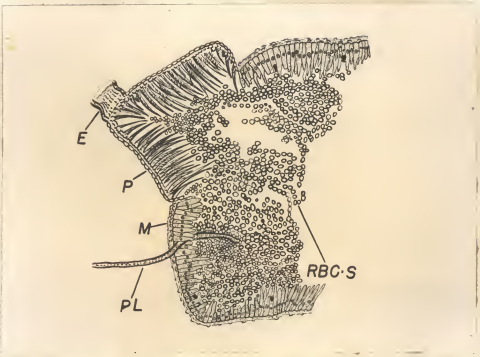
A, anus; AG, abdominal ganglion; AP, aspiratory pharynx; BC, buccal cavity; BCX, bursa copulatrix; DS, duct of spermatheca; E, esophagus; EG, brain (supra and subesophageal ganglion); EP, epipharynx; H, heart; HP, hypopharynx; LP, labial palps; M, midgut; MN, mandibles; MT, malpighian tubule; MX, maxilla; MXP, maxillary palps; O, ovary; P, proventriculus; R, rectum; RG, rectal gland; S, spermatheca; SD, salivary duct; SG, salivary gland; SP, salivary pump; TG, thoracic ganglion; U, uterus; V, vulva.

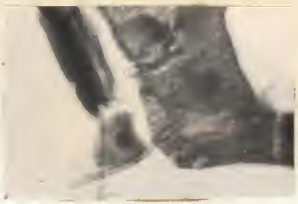
PLATE V



Sagittal section of a flea showing muscle systems and haemocoeles of head, thorax and abdomen. Notice fat body cells in posterior region of abdomen.

PLATE VI





Prelarva moving out of midgut of a flea into the haemocoele of the abdomen.

Abbreviations

E, esophagus; M, midgut; P, proventriculus; PL, prelarva; RBC.S, blood meal.

PLATE VII

Figure 7

Figure 8

Figure 7. Prelarvae in the midgut of a flea.

Figure 8. Photomicrograph of a sausage stage larvae in a drop of physiological saline solution.

PLATE VIII

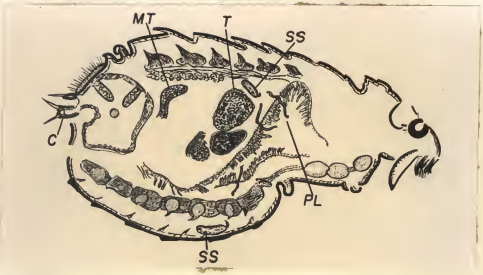


Figure 9

Figure 10

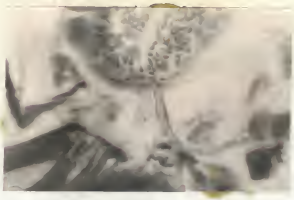
Figure 9. Prelarvae moving out of midgut and sausage stage larvae in the haemocoele of the abdomen of a Ctenocephalides canis male.

Figure 10. Photomicrograph of a sausage stage larva in the haemocoele of the abdomen (saline dissection).

Abbreviations

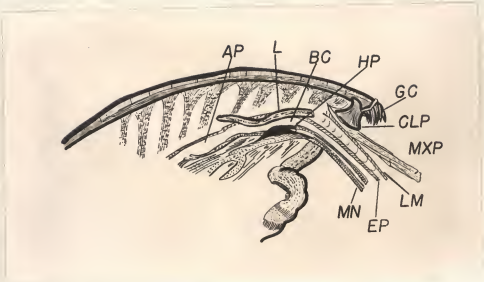
C, clasper; MT, malpighian tubules; PL, prelarva; SS, sausage stage larva; T, testes.

PLATE IX



Third stage larvae (L) moving from the haemocoele of the abdomen into the haemocoele of the thorax of a flea.

PLATE X



Serial section of the head and mouthparts of Ctenocephalides felis showing part of an infective stage larva in the vicinity of the mouthparts.

Abbreviations

AP, aspiratory pharynx; BC, buccal cavity; CLP, clypeus; EP, epipharynx; GC, genal comb; HP, hypopharynx; L, infective stage larva; LM, labrum; MN, mandibles; MXP, maxillary palps.

LITERATURE CITED

Augustine, D. L. 1938. Observations on the occurrence of heartworms Dirofilaria immitis (Leidy, 1856) in New England dogs. Amer. Jour. Hyg., 28 (3): 390-395.

Bancroft, T. L. 1901 Preliminary notes on the intermediary host of Filaria immitis, Leidy. Jour. & Proc. Royal Soc. of New S. Wales, (35): 41-48.

Blacklock, D. B. 1926. The development of Onchocerca volvulus in Simulium damnosum. Ann. Trop. Med., 20: 1-48.

Blackberg, S. N. and R. Ashman. 1930. Jour. Amer. Vet. Med. Assoc., LXXVII: 204-211.

Bobe-Moreau, A. 1813, Jour. Gen de Med. de Sedillot, XLVII: 3.

Bradley, R. E. 1952. Observation on the development of Dirofilaria immitis in certain insects. Tenn. Acad. Sci., 27 (3): 206.

_____. 1953. Observations on the development of Dirofilaria immitis in mosquitoes. Tenn. Acad. of Sci., 28 (3): 176.

Breinl, A. 1921. Preliminary note on the development of the larvae of Dirofilaria immitis in dog fleas Ctenocephalides felis and canis. Ann. Trop. Med. & Parasit., 14: 389-392.

Brown, H. W. 1939. Observation on the dog heartworm Dirofilaria immitis. N. Amer. Vet., 20 (1): 49-55.

Burch, G. and H. E. Blair. 1951. A rapid test for diagnosis of Dirofilaria immitis. Vet. Med., 46 (4): 128-130.

Connal, A. and S. L. M. Connal. 1922. The development of Loa loa (Guyot) in Chrysops silacea (Austen) and Chrysops dimidiata (van der Wulp). Tr. Roy. Soc. Med. & Hyg., 16: 64-89.

Del Rosario, F. 1936. Dirofilaria immitis and its culicine intermediate hosts in Manila, Philippine Islands. Philippine Jour. Sci., 60: 45-55.

Feng, L. C. 1930. Experiments with Dirofilaria immitis and local species of mosquitoes in Peiping, North China, with a note on Lankesteria culicis found in Aedes koreicus. Ann. Trop. Med. & Parasitol., 24: 347-364.

Fox, I. 1940. Fleas of the Eastern United States. (Ames, Iowa, State College Press), pp. i-vii + 191.

Grassi, B. 1888. Beträge zur kentniss des entwicklungscyclus von fünf parasiten des hundes. Centralbl. f. Bakter. u. Parasitenk., IV: 609, 776.

Grassi, B. and S. Calandruccio. 1890. Ueber hasmatozoen Lewis. entwicklungscyclus einer Filaria (Filaria recondita Grassi) des hundes. Centralbl. f. Bakter. u. Parasitenk., VII: 18.

Grassi, B. and G. Noe. 1900. The propagation of the filarias of the blood exclusively by means of the puncture of peculiar mosquitoes. Brit. Med. Jour., 2: 1306-1307.

Gray, P. 1952. Handbook of basic microtechnique. Philadelphia (Blakiston Co.), pp. i-viii + 141, 57 figs.

Hall, M. C., K. W. Price and W. H. Wright. 1934. Parasites and parasitic diseases of dogs. U. S. Dept. Agr. Circ. 338, p. 4.

Hinman, E. H. 1935. Experimental studies on filarial periodicity. Jour. Trop. Med. & Hyg., 38 (21): 265-267.

_____. 1935. Studies on the dog heartworm, Dirofilaria immitis, with special reference to filarial periodicity. Amer. Jour. Trop. Med., 15 (3): 371-383.

Hinman, E. H. and D. D. Baker. 1936. Jour. Trop. Med., 39: 101.

Hu, S. M. K. 1931. Studies on host-parasite relationships of Dirofilaria immitis Leidy and its culicine intermediate hosts. Amer. Jour. Hyg., 15 (3): 614-629, 5 figs.

La Peyronnie, M. 1778. (Quoted by Senac 1778) Tr. de Malad Coeur., 2nd ed., vol. I: 241.

Leidy, J. 1856. Synopsis of Entozoa. Proc. Acad. Natural Soc., Phila.

_____. 1881. Proc. Acad. Natural Soc., Phila.: 13.

Lewis, T. R. 1875. On nematode haematozoa in the dog. Quart. Jour. Microsc. Soc., New Series, XV: 268.

Manson, P. 1877. On Filaria Sanguinolenta and Filaria immitis. (Report on Haematozoa). Customs Gazette.

Megnin, A. 1883. Mem. Sur les Hematozoaires du Chien. Jour. de l'Anat. et de la Physiologie: 172.

Menon, T. B. and B. Ramamurti. 1941. The behavior of the infective larvae of Wuchereria bancrofti with special reference to their mode of escape and penetration of skin. Ind. Jour. Med. Res., 29: 393-401, 3 figs.

Panthot, J. 1679. Jour. des Savants. (Quoted by Neumann, L. G. 1892) A treatis on the parasites and parasitic diseases of the domesticated animals. New York and London: 250.

Patton W. S. 1913. A textbook of medical entomology. London (Christian Literature Soc. for India), pp. xvii + 764, 89 pls.

Peysson, A. 1806. Jour. de Med., Chir., Pharm. de Corvisart, XI: 441.

Phillips, J. H. 1939. Studies on the transmission of Dirofilaria immitis in Massachusetts. Amer. Jour. Hyg., 29 (3): 121-129.

Schuppert, A. 1858. New Orleans Med. News and Hospital Gazette: 680.

Silva Aranjo, R. 1878. Lyon Medical, XXIX: 319, 363.

Somerville, J. 1875. Customs Gazette No. 7, Shanghai.

Summers, W. A. 1940. Fleas as acceptable intermediate hosts of the dog heartworm Dirofilaria immitis. Proc. Soc. Exp. Bio. & Med., 43 (3): 448-450.

_____. 1943. Experimental studies on the larval development of Dirofilaria immitis in certain insects. Amer. Jour. Hyg., 37 (2): 173-178.

Slifer, E. H. and R. L. King. 1933. Serial sectioning of grasshopper eggs. Science, 78: 366-367.

Sonsino, P. 1888. Ricerche sugli ematozoi del cane. Atti della Soc. Tosc. di Sc. Naturali, X.

Stitt, E. R., P. W. Clough and M. C. Clough. 1945. Practical bacteriology, haematology and animal parasitology. Philadelphia (Blakiston Co.), pp. i-xiii + 961, 208 figs.

Underwood, P. C. and P. D. Harwood. 1939. Survival and location of the microfilariae of Dirofilaria immitis in the dog. Jour. Parasit. 25 (1): 23-33.

Yamada, S. 1927. An experimental study on twenty-four species of Japanese mosquitoes regarding their susceptibility as intermediate hosts for Filaria bancrofti Cobbold. Sc. Rep. Gov. Inst. Inf. Dis., Tokyo Imp. Univ., 6: 559-622.

Yen, C. H. 1938. Studies on Dirofilaria immitis Leidy with special reference to the susceptibility of some Minnesota species of mosquitoes to the infection. Jour. Parasitol., 24 (3): 189-205.

Zeviani, B. 1806. Mem. di Matem. e di Fisica della Soc. Italiani-Verona, XIV.

BIOGRAPHY

Edmund Bruno Stueben was born on April 22, 1920, in Cuxhaven, Germany. During the years from 1925 through 1933 he attended various elementary schools in New York and Cleveland, Ohio. In September 1937, he left Stuyvesant Science High School of New York and entered New York University. He majored in geology and received the degree Bachelor of Science in 1941. Upon completing college he joined the First Infantry Division of the United States Army. Following termination of service in October 1945 he joined the Bureau of Construction and later the Bureau of Plant Operation and Maintenance of the Board of Education, City of New York, as Assistant Mechanical Engineer. In September 1947 he entered Baylor University, Waco, Texas, for additional course study in biology. The following year he began his graduate study in biology and was awarded the degree Master of Arts in August 1949. In February 1950 he entered the University of Florida for graduate study in the field of biology. During the period of September 1951 until June 1953 he worked as a graduate assistant in the Department of Biology. He was awarded the degree Doctor of Philosophy in August 1953.

This dissertation was prepared under the direction of the Chairman of the candidate's Supervisory Committee and has been approved by all members of the committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council and was approved as partial fulfilment of the requirements for the degree of Doctor of Philosophy.

August 10, 1953

C. F. Byers
Dean, College of Arts and Sciences

R. E. Trueity
Dean, Graduate School

SUPERVISORY COMMITTEE:

Lewis Berner
Chairman

C. F. Byers

J. F. Slater

John H. Dawson

Archie Carr